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BRIEF COMMUNICATION

Biallelic mutations of *CFAP251* cause sperm flagellar defects and human male infertility

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Abstract

Multiple morphological abnormalities of flagella (MMAF) are human reproduction disorders due to the dysplastic development of sperm flagella. The spermatozoa of men with MMAF manifest absent, short, coiled, bent, and/or irregular-caliber flagella. Previous studies revealed genetic contributions to human MMAF, but known MMAF-associated genes only explained approximately 50% MMAF cases. In this study, we employed human whole-exome sequencing for genetic analysis and identified biallelic mutations of *CFAP251* (*cilia- and flagella-associated protein 251*, also known as *WDR66*) in three (5%) of 65 Han Chinese men with MMAF. All these *CFAP251* mutations are loss-of-function. The population genome data suggested that these *CFAP251* mutations are extremely rare (only heterozygous) or absent from human populations. Our functional assays of gene expression and immunofluorescence staining in a *CFAP251*-deficient man, together with previous experimental evidence from model organisms, suggested that CFAP251 is involved in flagellar functions. Our observations suggested that *CFAP251* is associated with sperm flagellar development and human male infertility.

Motile cilia and eukaryotic flagella are evolutionarily conserved organelles. Their dysfunction can cause many human diseases that are known as ciliopathies [1]. In mammals, the flagella, which are distinctive from the cilia participating in most types of body cells, are specifically developed to sperm tail during spermatogenesis with various motor and structure proteins [2]. The morphological alteration from spermatid must be made to power the spermatozoa motility [3, 4]. The abnormal expression or localization of any formation proteins of sperm flagella could cause male infertility. Multiple morphological abnormalities of flagella (MMAF) are human reproduction disorders due to the dysplastic development of sperm flagella [5]. The spermatozoa of MMAF patients manifest absent, short, coiled, bent, and/or irregular-caliber flagella (Fig. 1).

Previous genetic analyses in human MMAF have revealed pathogenic mutations in the genes encoding

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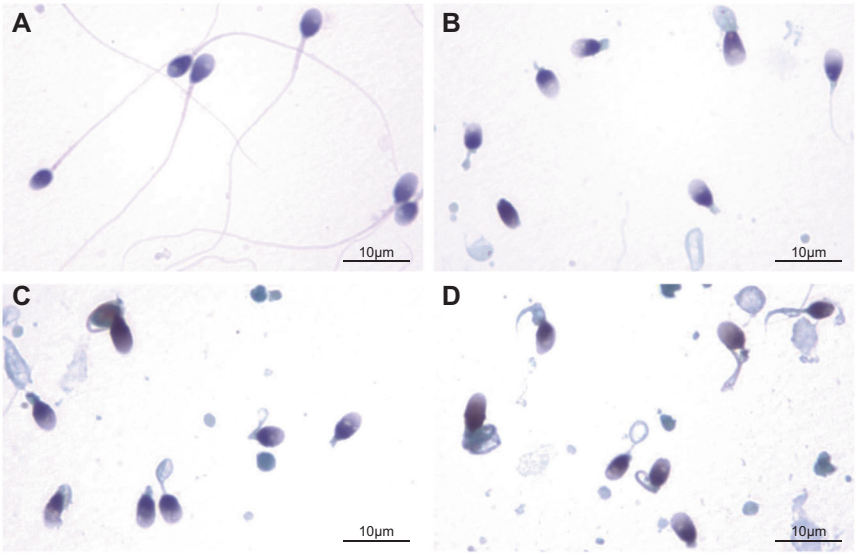

Fig. 1 Morphological study of the spermatozoa from MMAF cases under light microscopy. **a** Spermatozoa from a normal man showed smooth and elongated flagella with normal length. **b–d** The spermatozoa from *CFAP251*-deficient men presented with multiple morphological abnormalities of sperm flagella, including absent, short, coiled, bent, and/or irregular-caliber flagella in A009-IV-1 (**b**), A032-IV-1 (**c**), and S016-II-1 (**d**)

DNAH1 (dynein axonemal heavy chain 1) and three members of cilia- and flagella-associated proteins (CFAP43, CFAP44, and CFAP69) [5–8]. However, only approximately 50% cases of MMAF could be explained by these genes. The genetic causes and pathogenic mechanisms in the remaining unresolved MMAF cases still need to be further illustrated.

In this study, we proposed to identify pathogenic mutations by conducting human whole-exome sequencing (WES) of 65 Han Chinese men with MMAF. The patients were enrolled from the First Affiliated Hospital of Anhui Medical University and the Affiliated Suzhou Hospital of Nanjing Medical University in China. Intriguingly, we identified biallelic loss-of-function mutations in a novel MMAF gene *CFAP251* (also termed as *WDR66*, NM_001178003), which encodes the cilia- and flagella-associated protein 251. Our experimental observations suggested that the *CFAP251* gene is associated with sperm flagellar development and human male infertility.

All 65 cases from unrelated families were diagnosed as primary infertility with MMAF for years, and 19 of them were born in consanguineous families. No other reproduction problems such as ejaculation disorder, erectile dysfunction, and orchiatrophy could be detected. The chromosomal karyotypes of these cases were normal (46; XY) and there was no deletion detected in the human Y chromosome. In addition, primary ciliary dyskinesia-related symptoms of sinusitis, bronchitis, pneumonia, and otitis media were also excluded. This study was approved by the institutional review boards at Fudan University, Anhui Medical University, and Nanjing Medical University. Informed consent was obtained from all patients for being included in the study.

DNA samples were extracted from peripheral whole blood of all MMAF cases with the DNeasy Blood and Tissue Kit (QIAGEN). The WES analysis strictly followed the protocol of Agilent SureSelect^{XT} Human All Exon Kit. The Illumina HiSeq X-TEN platform was employed for DNA sequencing. The data analysis was conducted routinely on the human genome reference assembly (GRCh37/hg19) with the Burrows–Wheeler Aligner software [9]. PCR duplicates were removed and variant quality was evaluated by the Picard software (https://github.com/broadinstitute/picard). We called the DNA sequence variants using the Genome Analysis Toolkit [6, 10] and conducted functional annotation using ANNOVAR [11]. Previous studies suggest the autosomal recessive inheritance for human MMAF [5]. Therefore, homozygous or potential compound heterozygous mutations were investigated in this study. Loss-of-function mutations (such as stop-gain, stop-loss, frameshift, and splice-site mutations) and potential deleterious missense mutations predicted by SIFT, PolyPhen-2, and MutationTaster were obtained for further assessment. According to natural intolerance of male infertility, the MMAF-associated mutations cannot be common in the human populations. Therefore, two population genome databases, the 1000 Genomes Project [12] and ExAC [13], were used as references. Furthermore, the genes annotated with functional evidence of spermatogenesis were preferred [6].

Intriguingly, biallelic recessive mutations of *CFAP251* were identified in three (5%) of 65 human cases with MMAF (Fig. 2). All these mutations were verified by Sanger sequencing. A homozygous nonsense mutation, *CFAP251* c.799 C > T (p.Arg267*) was identified in the case A009-IV-1 (Fig. 2a). Only heterozygous carriers of this mutation have been found but are very rare in human

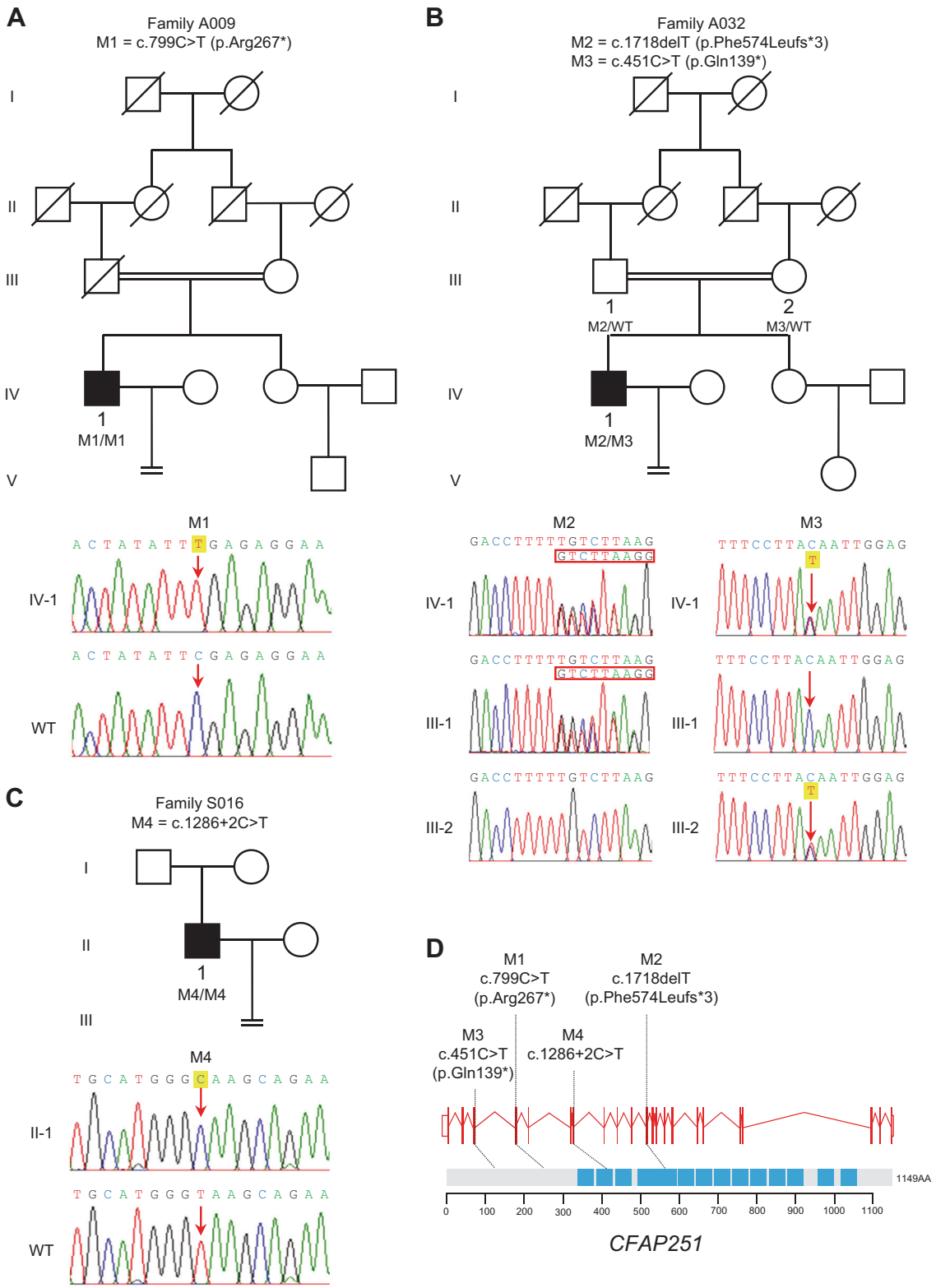

Fig. 2 Identification of biallelic *CFAP251* mutations in three probands with MMAF. **a–c** Biallelic *CFAP251* mutations were identified in the probands of three families. The mutations (M1–M4) identified by WES were further verified by Sanger sequencing. The red rectangles showed the shifted sequence in M2. **d** The positions of four *CFAP251* mutations (M1–M4) were indicated in the *CFAP251* gene and the protein domains of CFAP251. CFAP251 is rich in WD repeat domains. *WT* wild type, *AA* amino acid

populations; the mutant allele frequency is 8.5×10^{-6} in the ExAC database and absent from the human individuals of the 1000 Genomes Project (Supplementary Table 1). In the case A032-IV-1, compound heterozygous mutations were identified in *CFAP251* (Fig. 2b). One is a nonsense mutation c.415 C > T (p.Gln139*) and the other is a frameshift mutation, c.1718delT (p.Phe574Leu*3). Both *CFAP251* mutations were absent from more than 62 thousand individuals in the human populations archived in the 1000 Genomes Project and the ExAC database (Supplementary Table 1). Furthermore, a homozygous splice-site mutation c.1286 + 2 T > C was identified in the case S016-II-1 (Fig. 2c). This mutation is located at a conserved splicing donor site. It was also absent from the databases of 1000 Genomes Project and ExAC (Supplementary Table 1). The aforementioned mutations were predicted to cause premature translational termination of CFAP251 or alternate splicing donor site (www.umd.be/HSF3).

Both parents in family A032 were confirmed to be heterozygous carriers of *CFAP251* mutations (Fig. 2a). Unfortunately, DNA samples of the parents in families A009 and S016 were not available for genetic test. Here, we conducted further genetic assays to confirm the homozygous *CFAP251* mutations in the probands of families A009 and S016. Besides homozygosity suggested by Sanger sequencing, there is another possibility of hemizygous mutation, which could be caused by a deletion allele together with a point mutation at the same locus [14]. To exclude this possibility, we conducted high-density comparative genomic hybridization (CGH) microarray analysis in the probands of families A009 and S016. Notably, no obvious deletion or other copy-number variation was identified in *CFAP251* (Supplementary Figure 1), further suggesting that the *CFAP251* mutations in the individuals A009-IV-1 and S016-II-1 were homozygous mutations.

To investigate the pathogenic roles of the *CFAP251* mutations, we newly obtained the sperm sample of subject A032-IV-1. Real-time PCR (RT-PCR) assay was performed to investigate *CFAP251* mRNA expression. Total RNA was isolated following the protocol of Trizol reagent (Invitrogen) and was reverse-transcribed to complementary DNA (cDNA) with PrimeScript RT Reagent Kit (Takara). Eventually, we conducted RT-PCR using the qPCR SYBR Green Master Mix (Vazyme) with the internal control of *GAPDH* (Supplementary Tables 2 and 3). It was shown that the mRNA expressions of *CFAP251* were reduced dramatically in the *CFAP251*-deficient subject A032-IV-1 as compared to a male control (Supplementary Figure 2). Our observations suggested nonsense-mediated mRNA decay triggered by premature translation termination.

Protein expression of CFAP251 was also investigated by immunofluorescence staining. Human spermatozoa were fixed in 4% paraformaldehyde overnight. Subsequently, they were coated on the slides incubated with CFAP251 polyclonal antibody (Abnova) overnight and an hour incubation of AlexaFluor 488 anti-mouse (Yeasen) was operated at room temperature. Finally, slides were coated with Hoechst for 3 min. The signal of CFAP251 was lacking in the short sperm flagella of *CFAP251*-deficient man A032-IV-1, whereas the CFAP251 protein was clearly located in the whole sperm flagella of a normal man (Supplementary Figure 3). Both mRNA and protein levels of CFAP251 decreased or even disappeared, suggesting the deleteriousness of loss-of-function mutations in CFAP251.

Semen analysis was conducted according to the World Health Organization (WHO) guidelines. Semen samples of the *CFAP251*-deficient cases (A009-IV-1, A032-IV-1, and S016-II-1) were collected by masturbation after 2–7 days of sexual abstinence and evaluated after liquefaction for 30 min at 37 °C. The semen volumes and sperm concentrations of these cases were normal to the standards (Supplementary Table 1). However, the ratios of progressive motility were only 0.2%, 1.5%, and even zero in cases A009-IV-1, A032-IV-1, and S016-II-1, respectively. To assess sperm morphology under light microscopy, spermatozoa were stained by a modified Papanicolaou approach. The spermatozoa from these *CFAP251*-deficient men present flagellar defects with classical MMAF manifestation, including absent, short, coiled, bent, and/or irregular-caliber flagella as compared to the elongated sperm flagella from normal males (Fig. 1). To investigate the overall condition of sperm flagella in *CFAP251*-deficient cases, 200 spermatozoa were recorded for each case (Supplementary Table 1). Over 93% spermatozoa were affected with abnormal flagellar morphologies and most of them showed short, absent, and coiled flagella.

The *CFAP251* gene encodes an 1149 amino-acid protein (Q8TBY9). The gene expression of *CFAP251* is specific and preferential in testis according to the GTEx RNA-seq data of 8555 samples [15]. The CFAP251 protein contains 14 WD-domain repeats (WDR), often terminating in a Trp–Asp dipeptide that belongs to the WDR protein family [16]. Most WDR protein family members share an extremely conserved domain structure. The WDR protein family members, *CFAP43* and *CFAP44*, also contain nine WD repeats and genetically contribute to the pathogenesis of MMAF [6, 7]. WDR proteins are also known to serve as platforms for the assembly of protein complexes or mediators of transient interplay among other proteins. Plenty of molecular evidence and model organisms illuminate the importance of WDR proteins for axonemal structure of flagella.

FAP251, the ortholog of human CFAP251 protein in *Chlamydomonas reinhardtii*, was found as a component of cilium by proteomic analysis [17]. FAP251 in *Chlamydomonas* corresponded to CaM-IP4, which could be

precipitated by anti-CaM antibodies by using extracts isolated from wild-type axonemes [18]. Furthermore, it has been reported that a fraction of axonemal CaM is associated with radial spokes [19]. Cilium is formed with 96-nm repeat units, each of which contains three radial spokes (RS1, RS2, and RS3) with vital roles of transducting signals between the central pair of microtubules and inner dynein arms [20]. Through the arch-like structure containing DNAH1, RS3 stalk is connected to inner dynein arm 3 (IDA3). In *Dnah1*-knockout mice, the arch-like structure of the IDA3 was missing [21]. The arch-like structure density of the IDA3 was also lacked at the spoke base in *FAP251*-knockout *Tetrahymena thermophila* [22]. Furthermore, DNAH1 was predicted to co-express with CFAP251 by the STRING analysis [23]. Therefore, it is suggested that the impaired direct or indirect interaction of DNAH1 and CFAP251 could damage the connection between RS3 and IDA3. Additionally, the *FAP251* knockout strains of *Tetrahymena thermophila* showed that the swimming velocities reduced dramatically and the waveform of beating cilia was severely disturbed [22]. The immotility and dysplastic development of *FAP251*-knockout *Tetrahymena thermophila* cilia was also consistent with the MMAF manifestation of CFAP251-associated men.

Very recently, biallelic mutations of human *CFAP251* have been reported to induce male infertility. A homozygous ancestral SVA-insertion-mediated deletion in *CFAP251* was identified in the Tunisian cases with MMAF [24]. Furthermore, biallelic loss-of-function mutations of *CFAP251* were also identified in the cases with immotile spermatozoa [25]. The weakness and even loss of CFAP251 signal was also found in the *CFAP251*-deficient spermatozoa [25]. Furthermore, *Tetrahymena* manifested apparently immotility and decreased cell proliferation after 48 h of RNAi of *CFAP251* [24]. These recent independent observations further supported the roles of CFAP251 deficiency in abnormal flagellum organization and formation.

In conclusion, our experimental observations in Chinese MMAF cases suggested that biallelic loss-of-function mutations of *CFAP251* can result in sperm flagellar defects and male infertility. To fulfill comprehensive understandings of sperm flagellar development, the genetic analyses of human MMAF using WES and other genomic technologies will still be an efficient approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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